Levetiracetam treatment influences blood-brain barrier failure associated with angiogenesis and inflammatory responses in the acute phase of epileptogenesis in post-status epilepticus mice

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ARTICLE INFO
Keywords:
Levetiracetam
Blood-brain barrier
Epileptogenesis
Microglia
Angiogenesis
Status epilepticus

ABSTRACT
Our previous study showed that treatment with levetiracetam (LEV) after status epilepticus (SE) termination by diazepam might prevent the development of spontaneous recurrent seizures via the inhibition of neurotoxicity induced by brain edema events. In the present study, we determined the possible molecular and cellular mechanisms of LEV treatment after termination of SE. To assess the effect of LEV against the brain alterations after SE, we focused on blood-brain barrier (BBB) dysfunction associated with angiogenesis and brain inflammation. The consecutive treatment of LEV inhibited the temporarily increased BBB leakage in the hippocampus two days after SE. At the same time point, the LEV treatment significantly inhibited the increase in the number of CD31-positive endothelial immature cells and in the expression of angiogenic factors. These findings suggested that the increase in neovascularization led to an increase in BBB permeability by SE-induced BBB failure, and these brain alterations were prevented by LEV treatment. Furthermore, in the acute phase of the latent period, pro-inflammatory responses for epileptogenic targets in microglia and astrocytes of the hippocampus activated, and these upregulations of pro-inflammatory-related molecules were inhibited by LEV treatment. These findings suggest that LEV is likely involved in neuroprotection via anti-angiogenesis and anti-inflammatory activities against BBB dysfunction in the acute phase of epileptogenesis after SE.

1. Introduction
Levetiracetam (LEV) is an established second-generation anti-epileptic drug (AED) that exerts broad-spectrum anti-epileptic effects and is widely used to treat partial onset and generalized seizures (Lyseng-Williamson, 2011). In addition, LEV is a candidate second-line AED for status epilepticus (SE) (Manno, 2011; Glauser et al., 2016) and a candidate anti-epileptic drug (Pearl et al., 2013; Klein, et al., 2012). One of the pharmacological mechanism unique to LEV is its ability to bind to SV2A, a protein of the synaptic vesicle complex, to inhibit neurotransmitter release (Lynch et al., 2004; Meehan et al., 2012). In addition, several other mechanisms of LEV have been reported concerning the control of neurotransmitter release (Cataldi et al., 2005; Nagarkatti et al., 2008, Rigo et al., 2002; Lukyanetz et al., 2002).

Animal studies have shown that LEV exerts anti-epileptogenic and neuroprotective effects for the treatment of a pilocarpine (PILO)-SE model (Mazarati et al., 2004; Zheng et al., 2010; Itoh et al., 2015). However, the findings from previous reports in other post-SE animal models have been conflicting regarding whether LEV can prevent or modify epileptogenesis (Lösch et al., 1998; Glien et al., 2002; Klitgaard and Pitkanen, 2003; Stratton et al., 2003; Gibbs et al., 2006; Brandt et al., 2007). Furthermore, the mechanisms responsible for the anti-epileptogenic and neuroprotective effects of LEV are still unknown.

Post-brain insult epilepsy (post-traumatic, PTE; post-stroke, PSE;
and post-SE, PSEE; etc.) accounts for approximately 20% of symptomatic seizures and 5% of all epileptic seizures (Herman, 2002; Brodie et al., 2009). Given such prevalence, the prevention of these post-brain insult epilepsies is one of important issue. However, although 47 clinical studies have examined the efficacy of conventional AEDs (e.g., phenobarbital, valproate, carbamazepine, phenytoin, lamotrigine, topiramate), none of these drugs was able to prevent the development of epilepsy (Temkin, 2001; 2003; 2009; Krumholz et al., 2015). Therefore, several non-AEDs, such as anti-inflammatory drugs and mTOR inhibitors, were recently examined in basic and clinical studies to prevent these acquired epilepsies (Galanopoulou et al., 2012; Jiang et al., 2012). While LEV is an AED for managing post-brain insult epilepsies, several recent clinical studies for LEV in PTE, PSE, and PSEE have suggested that it may decrease the risk of acquired epilepsy or prevent the development of epilepsy, where conventional AEDs failed (Belcastro et al., 2008; Klein et al., 2012; Pearl et al., 2013). LEV has promising pharmacokinetic properties, including excellent bioavailability (>90%), linear kinetics, low plasma albumin binding (<10%), and a rapid rate of reaching steady state concentrations. In addition, LEV does not have any drug-drug interactions with AEDs or other agents that operate via the hepatic CYP-dependent metabolic pathway (Cloyd and Remmel, 2000; Patolsal, 2000; Panayiotopoulos, 2010).

We focused our attention on preventing the development of post-SE epilepsy. SE causes 3–5% of cases of symptomatic epilepsy; as such, SE patients are at a high risk of developing acquired epilepsy (Hesdorffer et al., 1998; Temkin, 2003; Jacobs et al., 2009). Various clinical trials have indicated that conventional AEDs suppressed acute seizures, but so far, none have been able to prevent the development of post-SE epilepsy (Temkin, 2001, 2003, 2009). Although the mechanisms underlying the relationship between SE and the development of epilepsy as part of the epileptogenic process are not well understood, the lack of efficacy of the conventional AEDs suggests that the biological mechanisms of the epileptogenic process may be differ markedly from that of the established epileptic brain models (Pitkanen et al., 2009).

Therefore, in the present study, we used a mouse model of PILO-induced SE as a model of epileptogenesis and investigated whether or not LEV treatment could protect against the SE-induced BBB failure associated with angiogenesis and brain inflammation in the latent period after SE.

2. Results

2.1. PILO-induced SE developed brain edema in MR images

In the MRI study, the SI of T2WI and DWI was measured in epileptogenic brains during the latent period after SE termination by DZP (Fig. 1A). At 2 days but not at 3 h post-SE, T2WI signal hyperintense areas were identified in the limbic regions (dorsal hippocampus, amygdala and piriform cortex) (Fig. 1A, b and c). In contrast, increased SI of DWI compared to the findings in pre-SE animals (Fig. 1A, e) was observed in the dorsal hippocampus and amygdala and piriform cortex at both 3 h and 2 days after SE (Fig. 1A, f and g).

In the quantitative MRI studies, the SI of T2WI and T2 values, and DWI and ADC were measured in the epileptogenic hippocampus during the latent period with or without LEV treatment. At 2 days but not at 3 h post-SE, increased SI of T2 and T2 values was inhibited by LEV treatment (Fig. 2B). In diffusion images, the SI of DWI increased at 3 h and 2 days post-SE, and the ADC decreased at 3 h, but increased at 2 days after SE. These changes in the diffusion of parameters returned to normal values following LEV treatment (Fig. 2C, D). These findings indicated that mice that had experienced consecutive SE five times developed cytotoxic edema in the early period (3 h after SE), and vasogenic edema sequentially appeared in limbic regions.

2.2. PILO-induced SE developed BBB failure in MR images

Vasogenic edema is generally characterized by an increase in the interstitial space fluid volume due to the loss of BBB integrity. To evaluate the loss of BBB integrity in vivo, the non-invasive nature of Gd-enhanced MRI using T1WI (GdET1WI) was employed. As shown in Fig. 1A (j and k), hyperintensity on GdET1WI was observed in the parenchyma of dorsal hippocampus 2 days after SE, but not 3 h after. However, 7 days after SE, the SI of GdET1WI significantly decreased to the pre-SE SI (Fig. 1A, I). Therefore, the observations in the acute phase of post-SE may have been due to cytotoxic edema where the BBB was still intact, whereas 2 days post-SE, vasogenic edema may have developed due to transient BBB failure. The enhancement of Gd-DTPA in the parenchyma of the limbic regions peaked at post-SE day 2 and subsequently decreased to normal SI at post-SE day 7 (Fig. 1A, I, Fig. 2E). However, after the occurrence of spontaneous recurrent seizures (SRSs), the enhancement of Gd-DTPA gradually increased week by week (Fig. 2E, F). Thus, the loss of BBB integrity exhibited biphasic responses in epileptogenesis, and the induced permeability of the BBB by SE was markedly ameliorated by LEV treatment (Fig. 2E). These results suggested that the development of the BBB by LEV treatment was associated with the protection of the BBB in the hippocampus. Therefore, in this study, we focused on LEV intervention for BBB failure during the acute phase of the latent period after SE. We hypothesized that preventing the acute process using LEV would regulate the appearance of SRS (spontaneous recurrent convulsive seizures) by controlling sequential molecular and cellular cascades.

2.3. Immunohistochemical studies of SE-induced BBB failure and reactive astrogliosis

To confirm the effect of LEV against BBB failure after SE, immunohistochemical studies were performed using anti-albumin and anti-GFAP antibodies in the hippocampus of PILO-SE mice with or without LEV treatment. As shown in Fig. 3A (b–d), albumin clearly diffused into the parenchyma of the hippocampus 3 h, 2 and 7 days after SE termination. However, in the LEV-treated mice, the diffusion of albumin into the parenchyma was strongly inhibited (Fig. 3A, f–h). Cerebral vessels were mainly covered by the endfeet of astrocytes to compose the BBB. GFAP-positive reactive astrogliosis appeared and persisted in hippocampal CA1 over time after SE (Fig. 3A, j–l). Reactive astrogliosis was inhibited by LEV treatment (Fig. 3A, n–p). In addition, the change in the GFAP mRNA expression correlated with SE-induced astrogliosis (Fig. 3B). Taken together, these findings indicated one possibility of protection of BBB via astrocytes by LEV treatment after SE termination.

2.4. Effect of LEV treatment on SE-induced angiogenesis in the hippocampus

To understand the mechanisms leading to the BBB leakage involved in SE-induced angiogenesis in the hippocampus, immunohistochemical studies were performed using anti-CD31 (PECAM-1) antibody to identify cerebral immature endothelial cells (Vittet et al., 1996), and the expression of angiogenesis-related molecules was measured pre-and post-SE. The number of CD31-positive cells gradually increased during the acute phase after SE, and this increase was inhibited following treatment with LEV for 2 days (Fig. 4A, B). The expression of angioptoid-2 (Ang-2) mRNA significantly increased at 3 h and 2 and 7 days after SE, and the expression of angioptoid-1 (Ang-1) mRNA significantly increased at 7 days after SE. In addition, the expression of the receptor Tie-2 mRNA showed biphasic changes, with a decreased expression at 1 and 3 h and an increased expression at 2 days after SE (Fig. 4C). LEV treatment prevented the increase in the expression of Ang-2 mRNA at 2 and 7 days after SE and in the
expression of Tie-2 mRNA at 2 days after SE; however, this treatment did not influence the expression of Ang-1 mRNA (Fig. 4C). The expression of Ang-2 protein (6.27 ± 2.06 pg/mg) at 2 days post-SE was significantly increased from the pre-SE level (1.64 ± 0.11 pg/mg), and its increased protein level at 2 days post-SE was significantly downregulated by LEV treatment (1.99 ± 0.0.78 pg/mg). The mRNA expression of another angiogenic factor, vascular endothelial growth factor-A (VEGF-A), and its receptor, VEGF-R2, also gradually increased and peaked at 2 days after SE; this increased mRNA expression was also significantly inhibited by LEV treatment (Fig. 4D). These findings indicated that the induction of angiogenesis in the acute phase of the latent period coincided with the time course of BBB leakage, and that these changes were inhibited by LEV treatment (Fig. 4D). These findings suggested that the inhibition of angiogenesis in the hippocampus by LEV treatment after SE might be another possible mechanism of BBB protection.

2.5. Morphological changes of the microglia in the post-SE hippocampus following LEV treatment

Neuronal cell death in hippocampal CA1 was clearly observed by cresyl violet staining at 2 days after SE, and the cell death was clearly protected by LEV treatment (Fig. 5). Recently, several studies have suggested that this cellular damage was involved in neuroinflammation via microglia activation (Marchi et al., 2014; Kosonowska et al., 2015). To examine the effect of LEV against microglia activation after SE, immunohistological studies were performed using anti-Iba1 and CD68 antibodies to identify microglia and phagocytotic microglia, respectively. After SE termination, the number of Iba1-positive microglia gradually increased in CA1 (Fig. 6, upper panels; w/o); however, this increase was dramatically inhibited by subsequent treatment with LEV (Fig. 6, bottom panels; w/LEV). The morphology of the Iba1-positive cells was a ramified-like shape at 3 h after SE, changing to an amoeboid-like shape 2 days after SE. CD68- and Iba1-double positive microglia appeared in the CA1 pyramidal layer 2 days after SE. The appearance of CD68-positive phagocytotic microglia in this region was coincident with neuronal cell death (Fig. 5). However, no phagocytotic microglia were observed in LEV-treated SE mice, because of the protective effects of LEV treatment against neuronal cell death. These results suggested that the relationship between microglia activation and neuronal cell death in the acute phase of epileptogenesis might play an important role in the development of epilepsy...

2.6. Effect of LEV on the post-SE increase in levels of pro-inflammatory cytokines in the hippocampus

As several studies reported that neuroinflammation was involved in CNS injuries in epilepsy (Vezzani et al., 2011, 2015), we determined the involvement of neuroinflammation after SE. To this end, we induced the temporal mRNA expression of pro-inflammatory related-
molecules, focusing on the early phase in experimental epileptogenesis. At 1 h post-SE, significant increase in the levels of pro-inflammatory cytokine mRNA IL-1β (peaked 3 h), IL-6 (peaked 2 days), TNFα (peaked 2 days), and iNOS (peaked 1 h)—were observed compared to pre-SE (Fig. 7A, D, E, F). The protein levels of pro-inflammatory cytokines IL-1β (peaked 3 h, 6.68 ± 1.23 pg/mg) and IL-6 (peaked 2 days, 209.19 ± 83.64 pg/mg) were markedly post-SE than pre-SE (IL-1β, 1.95 ± 0.31 pg/mg; IL-6, 0.19 ± 0.01 pg/mg) as well. The expression kinetics of IL-1R type I (IL-1R1) mRNA and Toll-like receptor 4 (TLR4) activated by IL-1β peaked 3 h and 2 days, respectively, after SE (Fig. 7B, C). These results indicated that induction of pro-inflammatory-related molecules occurred in the order of iNOS, IL-1β/IL-6/IL-1R1, and TNFα/TLR4, according to the highest expression levels. These sequential mRNA changes in pro-inflammatory molecules in the early phase after SE were significantly attenuated by LEV treatment (Fig. 7), as were the protein levels of pro-inflammatory cytokines IL-1β (3 h; 3.24 ± 0.20 pg/mg), IL-6 (2 days; 0.72 ± 0.50 pg/mg) post-SE. These sequential increases in the
levels of pro-inflammatory molecules in the acute phase of post-SE were normalized to non-inflammatory level following LEV treatment.

3. Discussion

The ZP prevented BBB failure associated with angiogenesis and neurodegeneration induced by inflammatory responses in PILO-SE.
LEV, one of the newer AEDs, has a unique mechanism of action, wide therapeutic spectrum, and a favorable pharmacokinetic profile (Panayiotopoulos, 2010). In addition, this drug may also prevent or modify the development of acquired epilepsy in basic and clinical studies (Lösch et al., 1998; Klitgaard and Pitkanen, 2003; Belcastro et al., 2008; Klein et al., 2012; Pearl et al., 2013). However, several reports have shown that LEV did not prevent acquired epilepsy when administered in post-SE animal models of TLE, although it did exert neuroprotective effects (Gibbs et al., 2006; Brandt et al., 2007). The

![Fig. 4. LEV treatment prevented SE-induced angiogenesis in the hippocampus.](image)

**A.** The immunohistochemistry of CD31 for immature endothelial cells in mice treated without (a, b) or with (c) LEV 2 days after SE (post-SE day 2). **B.** The box plots of the number of CD31-positive immature endothelial cells in the dorsal hippocampus (cells/mm²) show the 25th and 75th percentiles as the upper and lower half of each box, with the 10th and 90th percentiles as the upper and lower error bars plus individual outliers (n=3–5). The number of CD31-positive cells per mm² in hippocampal CA1 area measured 2 days after PILO-induced SE (post-SE), and before PILO (pre-SE; blue bar) with (+; pink bar) or without LEV (w/o; green bar) treatment in mice. ††; p < 0.01 vs. pre-SE, †; p < 0.05 vs. w/o post-SE. **C.** The box plots of the relative expression of angiopoietin-1 (Ang-1, upper panel), angiopoietin-2 (Ang-2, middle panel) and its receptor Tie-2 mRNA (bottom panel) in the hippocampus show the 25th and 75th percentiles as the upper and lower half of each box, with the 10th and 90th percentiles as the upper and lower error bars plus individual outliers (n=3–8). The levels of Ang-1, Ang-2 and Tie-2 mRNA (D) were measured 1 and 3 h and 2 and 7 days after PILO-induced SE (post-SE), and before PILO (pre-SE; blue bar) with (+; pink bar) or without (−; green bar) LEV treatment in mice. †; p < 0.05, ††; p < 0.01 vs. pre-SE, *; p < 0.05, **; p < 0.01 vs. w/o post-SE.
discrepancy in these findings regarding the effects of LEV in post-SE animal models were likely caused by the differences in the study design.

Fig. 5. LEV treatment prevented neuronal cell loss after PILO-induced SE in mice. Typical micrographs of cresyl violet-stained coronal sections showing a pre-SE, and a PILO-SE mouse treated with vehicle (post-SE, w/o) or with 350 mg/kg LEV at 3 h, 2 days, and 7 days after SE (post-SE, w/LEV). Magnifications of CA1, CA3, and DG in each hippocampal section are shown in the insets. The arrows indicate the cresyl violet-stained pyramidal layer in dorsal hippocampal CA1.

Fig. 6. Effect of LEV treatment on Iba1- and CD68-positive microglia in post-SE hippocampal CA1. Typical double-immunofluorescence micrographs showing co-localization of CD68 in Iba1-positive microglia in the dorsal hippocampal CA1 region. The upper left panels show sections labelled with an antibody against CD68 (green), the bottom left panels show sections labelled with an antibody against Iba1 (red), and the bottom right panels show sections double-labelled with antibodies against CD68 and Iba1 (yellow signal in post-S2 days without LEV) (typical microglial form in inset). The upper right panels show counter-stained sections using DAPI-Fluoromount-G for DNA detection. The coronal sections of pre-SE and PILO-SE mice treated with vehicle (post-SE, w/o) or with 350 mg/kg LEV (w/LEV) at 1 h, 3 h, and 2 days after SE (post-SE).
(e.g. dose, timing and duration of intervention, severity of SE, including the number of seizures, and kind of animal models, including species and strains) (Dudek et al., 2008).

In our previous study, we reported that the incidence of SRS after PILO (290 mg/kg) induced 5 consecutive seizures was inhibited by LEV treatment, and mortality, brain edema, and neuronal cell loss after SE were also prevented (Itoh et al., 2015). The severity of SE, which was determined based on the dose of PILO and the number of seizures, was important for assessing the effect of LEV. Indeed, high doses of PILO ( > 350 mg/kg, i.p.) or more than 6 consecutive seizures resulted in poor outcomes, including mortality, brain edema (MRI), and BBB disruption (Fig. 1S, Tables 1S–3S). In addition, LEV was not as effective in models induced at lower doses of PILO ( < 290 mg/kg) or with fewer than 3 consecutive seizures (Tables 1S and 2S), and when administered more than 2 d post-SE (Fig. 2S). The severity of SE and timing of LEV intervention after SE were found to be important factors influencing the efficacy of LEV.

DZP is commonly used to treat SE, especially in Japan. It is well-known in the SE model that convulsive SE was terminated by DZP, but nonconvulsive SE was not. To attenuate nonconvulsive SE after DZP treatment, the addition of continuous LEV treatment was found to be effective (Mazarati et al., 2004). A recent report indicated that interictal spiking and high-frequency oscillation during post-SE occurred at significantly lower rates following LEV plus DZP treatment compared to DZP-only controls (Lêvesque et al., 2015). Therefore, the LEV treatment after SE probably decreases or prevents the acute molecular and cellular alterations resulting from nonconvulsive SE in addition to convulsive SE.

3.1. SE-induced angiogenesis associated with BBB leakage against LEV

The potential mechanisms of LEV may involve the protection against BBB leakage via angiogenesis after SE. Indeed, BBB phasic failure after SE was coincident with morphological changes of the associated cells of BBB, especially astrocytes and endothelial cells.

Changes in the morphology and function of astrocyte in response to brain insults are collectively termed “reactive astrogliosis”, although astrocytes are essential for the maintenance of the cytoarchitecture and homeostasis under normal brain conditions (Sofroniew and Vinters, 2010). LEV treatment inhibited GFAP-positive astrogliosis after SE and SE-induced angiogenesis (Figs. 3 and 4). Reactive astrogliosis is associated with BBB failure and may trigger angiogenesis (Kovács et al., 2012; Morin-Brureau et al., 2012). Angiogenesis, the formation of new blood vessels, is induced by various growth factors and cytokines that act either directly or indirectly. In the present study, the expression of VEGF and VEGF-R2, which are involved in angiogenesis and vascular permeability (Pages and Pouyssegur, 2005), was significantly upregulated at 2 days after SE (Fig. 4D). A previous study proposed that the upregulation of VEGF-A after SE depends on hypoxia-inducible factor (HIF1-α), as SE causes hypoxia via a decrease in the cerebral blood flow (Rigau et al., 2007). However, given that we did not detect any upregulation of HIF1-α mRNA in the acute phase (1 or 3 h and 2 days) (Fig. 4S), the upregulation of VEGF may have
instead been induced by other factors, such as pro-inflammatory cytokines (Pages and Pouyssegur, 2005; Vezzani and Granata, 2005). Indeed, in the present study, the expression of TNF-α, IL-1β, and IL-6, were markedly upregulated in the acute phase after SE compared with pre-SE (Fig. 7).

Angiopoietin-1 (Ang-1)/its receptor Tie-2 control vessel maturation and maintain vasculature, while Ang-2/its receptor Tie-2 destabilize vessels and promote angiogenesis (Maisonpierre et al., 1997). As shown in Fig. 1A, l and Fig. 2E, the SI of GdET1WI was significantly increased at 2 days post-SE and subsequently decreased to the pre-SE level by 7 days post-SE. A concomitant marked increase in Ang-2 expression was also noted at the gene and protein levels, raising the possibility that Ang-2 may play a role in BBB failure in the acute phase of post-SE (Fig. 4C). However, the expression of Ang-1 was actually upregulated at 7 days after SE but not in the acute phase, until 2 days post-SE (Fig. 4C). These results suggested that Ang-1 and Ang-2 have reciprocal effects for angiogenesis during the latent period of epileptogenesis. In addition, the Ang-2/Tie2 cascade regulated the early steps of angiogenesis associated with one of more angiogenic factors, the VEGF-A/VEGF-R2 cascade (Lobov et al., 2002; Scholz et al., 2015). Upregulation of Ang-2/Tie2 and VEGF-A/VEGF-R2 therefore leads to BBB leakage via angiogenesis (Morin-Brureau et al., 2012; Nag et al., 2005).

Some studies have shown that BBB injury is part of a pathophysiological process affecting the neovasculature via seizures in brain (Rigau et al., 2007). The BBB leakage after SE may have several causes. One possible cause is that the morphological changes of the astrocytes observed after SE transiently damage the functional BBB architecture, causing leakage. Another possible cause is the initial angiogenic processes due to the migration and proliferation of endothelial cells induced by angiogenic factors such as Ang-2/Tie2 and VEGF-A/VEGF-R2 cascades, as the vascular leakage was caused by neovascularization without the BBB functional architecture.

In the present study, we showed that LEV treatment protected against the transient breakdown of BBB due to angiogenic processes (Figs. 2–4). Although how LEV induces these protective effects is unknown, one possibility is that LEV may be associated with the prevention of SE-induced brain edema, which would consequently lead to protection against BBB leakage associated with angiogenesis. Therefore, we hypothesize that LEV treatment prevents the development of epilepsy as a result of protection against the angiogenesis associated with an immature BBB, although LEV treatment did not enhance the expression of Ang-1 to antagonize the effect of Ang-2. LEV may affect angiogenic processes such as Ang-2/Tie2 and VEGF-A/VEGF-R2 cascades in the neovascularature associated with reactive angiogenesis, although further studies will be required to resolve the detailed mechanism.

3.3. SE-induced activation of microglia against LEV treatment

Recent emerging reports on basic and clinical studies have focused on the role of microglia in epilepsy, as excessive microglia activation may be involved in brain inflammation, contributing to the pathophysiological process of epilepsy (Vezzani et al., 2015). The brain inflammation induced by activated microglia in the BBB may compromise barrier function and subsequently kill neurons, in addition to angiogenesis as described above.

As shown in Fig. 6, morphological changes to the microglia were observed in the hippocampus after PILO-induced SE. Recently, microglial activation has been suggested to proceed in three phases with respect to the morphology and function (Sajjo and Glass, 2011; Kettenmann et al., 2011). Ramified shape microglia converted to an activated microglia accompanying partial round shape at 3 h after SE, and then sequentially changed to an amoeboid-like shape 2 days after SE (Fig. 6). The activated microglia produce many pro-inflammatory molecules, such as cytokines, chemokines, and reactive oxygen/nitrogen species (Brown and Neher, 2010; Vezzani et al., 2015). In the present experimental model, in the acute phase at 1 or 3 h after SE, the expression of pro-inflammatory molecules such as TNF-α, IL-1β, IL-6, and iNOS increased markedly, accompanying morphological activation (Figs. 6 and 7). LEV prevented the increase in the numbers of Iba1-positive microglia and the conversion to an amoeboid-like shape from a ramified shape after SE, as well as significantly inhibited the expression of pro-inflammatory molecules (Figs. 6 and 7).

In general, excess pro-inflammatory molecules are harmful to neuronal cells (Brown and Neher, 2010). Therefore, these findings suggest that neuronal cell death was induced by excess pro-inflammatory molecules generated by microglia, and that LEV protects neurons by preventing the production of pro-inflammatory molecules via the inhibition of SE-induced microglia activation. Although we did not directly investigate the crosstalk between the activated microglia and astrocytes, the production of pro-inflammatory molecules by activated microglia enhances the drive of pro-inflammatory molecules from reactive astrocytes, which further enhance the activation of the microglia via a positive feedback pathway.

As mention above, these findings suggest that the transient BBB leakage after SE may also be caused by such crosstalk. Therefore, although the functional significance of the microglial and astrocyte crosstalk in vivo remains to be defined, the amplification of brain inflammation by this crosstalk is important for understanding the mechanism of epileptogenesis and the effects of LEV.

Microglia showed a CD68-positive phagocytic phenotype at day 2 post-SE but not 3 h after SE and congregated in hippocampal CA1 (Fig. 6). Given that the loss of pyramidal neurons in the hippocampal CA1 of PILO-treated mice occurred 2 days after SE, the microglia changed to a phagocytic phenotype due to the clearance of the debris. Almost no reports have clearly shown the relationship between pathogenesis and microglial phagocytosis, although a phagocytic reaction is generally an adaptive response to remove bacteria or debris that are not required for survival. Pro-inflammatory molecules appear to induce neuronal loss via microglial activation and phagocytosis, causing neuronal death by phagocytosis (Neniskyte et al., 2014). Given that LEV treatment prevented SE-induced microglial activation and neuronal cell death, CD68-positive phagocytic microglia consequently might not need to congregate.

However, we cannot rule out the permeability of blood-borne cells, including phagocytic cells such as macrophages as well as neutrophils, through the damaged BBB or neovascularization without a mature BBB, since it is difficult to distinguish between microglia and macrophages. In the present study, given that Iba1/CD68 double-positive cells existed in the perineovasculature, some of these cells might have been blood-borne phagocytic cells. Nevertheless, the LEV treatment-induced protection against BBB leakage due to angiogenesis and brain inflammation may have prevented any attack by blood-borne phagocytic cells. Future studies should investigate whether LEV directly or indirectly inhibits SE-induced microglia activation by protecting neurons, and the significance of the suppression of microglial activation by LEV should also be examined to reveal the cellular and molecular mechanisms of LEV in epileptogenesis and subsequent SRS.

Brain damage following TBI and stroke may develop via mechanisms similar to that of brain injury following SE (Donkin and Vink, 2010; Manley et al., 2000). Therefore, the development of post-brain insult epilepsies may be associated with BBB failure due to brain inflammation, and LEV treatment may suppress the brain inflammatory process after these insults. Further prospective evaluations are needed to elucidate the molecular and cellular mechanisms of action for inhibiting angiogenesis, brain inflammation, and brain edema underlying repeated LEV treatment for protecting against BBB leakage.
in the acute phase of the latent period. In conclusion, we found that LEV inhibited SE-induced angiogenesis and microglia activation, and the novel mechanism of LEV normalized the abnormalities in the transient BBB breakdown during the acute phase of the latent period after SE. However, the molecular mechanisms of the cerebrovascular and parenchymal homeostatic mechanisms during epileptogenesis remain poorly understood. The present findings indicated that the development of BBB leakage due to brain inflammation and angiogenesis is critical in the initial process of developing acquired epilepsy after SE. Clarifying the effects of LEV treatment on the balance between the damage and recovery processes during the acute phase post-SE will open new avenues for understanding the mechanism of acquired epilepsy and may be key to developing new therapies.

4. Experimental procedures

4.1. Experimental animals

The protocols for all animal experiments were approved by the Tokushima Bunri University Animal Care Committees and were performed in accordance with the National Institutes of Health (USA) Animal Care and Use Protocol. All efforts were made to minimize the number of animals used and their suffering. Male, eight-week-old ICR mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained with laboratory chow and water ad libitum on a 12-h light/dark cycle. The utilized animals were euthanized using saturated KCl.

4.2. PILO-induced SE model and seizure assessment

The PILO-induced SE model is a well-studied animal model of SE-based acquired epilepsy, as temporal lobe epilepsy (Cavalheiro et al., 1996). After a designated fasting period (16 h), ICR (CD-1) mice (9–10 weeks old; weighing 35–45 g, Shizuoka, Japan) were injected with methyl scopolamine (1 mg/kg, Sigma Aldrich, St Louis, MO, USA) intraperitoneally [i.p.] in 0.9% NaCl (Otsuka Pharmaceutical Factory, Inc. Tokushima, Japan) 30 min prior to PILO. A single dose of PILO (Sigma Aldrich) was then administered (290 mg/kg, i.p. in 0.9% NaCl) (Itoh et al., 2015, Fig. 1S). The animals were placed in a plastic chamber (10×15×30 cm), and their behavior was observed before and thereafter when needed. Mice were placed in individual blocks in a plastic chamber, and their seizures were monitored and recorded using a rehydration solution (OS-1®; Takeda Pharmaceutical Ltd. Osaka, Japan) every day for 24 h, for 7–10 days after SE. The post-SE care of the animals included oral rehydration therapy to normalize the abnormalities in the transient BBB breakdown during the acute phase post-SE will open new avenues for understanding the mechanism of acquired epilepsy and may be key to developing new therapies.

4.3. Administration of Levetiracetam

The doses of LEV (LKT Labs, Inc., St. Paul, MN, USA) used in this study were 180 and 360 mg/kg. LEV dissolved in distilled water was orally administered at an injection volume of 0.1 mL/10 g of body weight within 30 min after DPZ injection, and thereafter twice a day (at 8:30 and 17:30) for 7–10 days. To determine the pharmacokinetics of LEV, the concentrations of LEV in the plasma and hippocampus were measured by HPLC at 0, 5, 1, 2, 3, 8, 12 and 24 h after a single injection of LEV (180 mg/kg, 360 mg/kg, p.o.) (Fig. 3S).

4.4. Magnetic resonance imaging (MRI)

The all mice who developed SE were anesthetized with a 1.5–1.8% isoflurane (Escaïn®, 160 mL/min: MERCK, Kenilworth, NJ, USA)-oxygen mixture. During MRI, the body temperature was measured using a rectal thermocouple and maintained at a constant 37 ± 0.2 °C with a feedback-controlled warm-water blanket (Yamashita Tech System, Tokushima, Japan) connected to the rectal probe (Photon Control Inc., Burnaby BC, Canada). The MRI data were acquired using an MRmini-SA (DS Pharma Biomedical, Osaka, Japan), consisting of a 1.5-Tesla permanent magnet made of Nd-Fe-B material, a compact computer-controlled console, and a solenoid MRI coil with a 30-mm inner diameter.

We obtained coronal MR images in all SE survivors using T1-weighted imaging (T1WI), T2-weighted imaging (T2WI), and diffusion-weighted imaging (DWI) sequences, in accordance with our previous report (Itoh et al., 2015). To measure the T2 relaxation time and apparent diffusion coefficient (ADC) from T2WI and DWI, respectively, in the hippocampus on a brain slice (bregma −1.70 to −2.06 mm) according to the mouse brain atlas (Faxins and Franklin, 2012) (Fig. 1y) were defined as regions of interest (ROIs) in the dorsal hippocampus, and the mean value of the signal intensity (SI) in the three ROIs of six animals treated with or without LEV at each time point was determined using the INTAGE Realia Professional software program (Cybernet Systems Co. Ltd., Tokyo, Japan).

To investigate the BBB permeability after SE, all SE survivors were bolus injected via a femoral vein with 0.4 mmol/kg Gd-DTPA (gadopentetate dimeglumine, Magnevist®; Bayer HealthCare LLC, Leverkusen, Germany) as an ionic gadolinium complex MRI contrast agent. Since Gd complex MRI contrast agents such as Gd-DTPA cannot cross the intact BBB, this agent does not accumulate in the normal brain parenchyma (Ichikawa and Itoh, 2011; Danjo et al., 2013; Itoh et al., 2015). Therefore, any accumulation of T1SI in the brain parenchyma indicates BBB leakage. The MRI observations were represented as typical micrographs of the first SRS-induced mice at day 7 after SE. However, the quantitative MRI parameters were included with the data of the first SRS-induced mice between days 7 and 10 after SE.

4.5. Total RNA extraction and real-time polymerase chain reaction (RT-PCR)

Determination of the mRNA levels was performed in accordance with our previous report (Ishihara et al., 2015). Briefly, total RNA was extracted from the mouse whole hippocampus using a High Pure RNA Isolation Kit (Roche Diagnostics K.K., Tokyo, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA following the ReverTra Ace protocol (Toyobo, Osaka, Japan) with a random primer (9-mer; Takara Bio, Shiga, Japan). RT-PCR was performed using a LightCycler instrument (Roche Diagnostics) in a total reaction mixture volume of 20 μL containing 10 μL of Sybr Green Real-time PCR master mix (Toyobo), 1 μL cDNA, and 10 pmol of each of the primers, as shown in Table 1. The levels of target mRNA were normalized to the β-actin manually reviewed by well-trained investigators.

Table 1. The levels of target mRNA were normalized to the β-actin...
mRNA level, and then the values of the treated slices were divided by those of the control group to calculate the relative mRNA levels (Schmittgen and Livak, 2008). The relative expressions of mRNA were included with the data of the first SRS-induced mice between days 7 and 10 after SE.

4.6. Histological, immunohistochemical, and stereological analyses

For the histological and immunohistochemical analyses, three mice for all groups were deeply anesthetized and euthanized with sodium pentobarbital (50 mg/kg; Sigma Aldrich) and then perfused transcardially with heparinized 0.1 M phosphate-buffer saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. The fixed tissues were embedded in paraffin and sectioned on a microtome at 4 µm for the immunohistochemical examination. The sections were incubated in 0.3% hydrogen peroxide in PBS for 30 min and then in 2% bovine serum albumin (BSA) for 30 min, followed by overnight incubation with a rabbit antibody for GFAP (1/400; Dako Agilent Technologies, Glostrup, Denmark), or mouse albumin (1/1000; MP Biomedicals, LLC- Cappel, Santa Ana, CA, USA). Staining was achieved by incubation with a Simple Stain kit (Nichirei, Tokyo, Japan) at room temperature for 5 min and sectioned on a microtome at 50-μm-thick and sectioned on a microtome at 50-μm-thick for the immunohistochemical examination. The sections were counterstained with hematoxylin.

For immunofluorescent histochemistry, the brains were removed from three mice for all groups, post-fixed overnight in 4% buffered PFA at 4 °C after perfusion, and then cryoprotected in 30% sucrose. The brain was frozen in powdered dry ice, and 50-µm-thick floating sections were prepared using a Cryostat (CM3050 S; Leica Biosystems, Nussloch, Germany). The sections were blocked and permeabilized with PBS including 10% normal goat serum (Sigma Aldrich) and 0.3% Triton-X 100 for 1 h at room temperature. The sections were then incubated with primary antibody (Anti-Iba1, 1/500; Wako Pure Chemical Industries, Ltd. Osaka, Japan), or mouse albumin (1/1000; MP Biomedicals, LLC- Cappel, Santa Ana, CA, USA). Staining was achieved with a Simple Stain kit (Nichirei, Tokyo, Japan) and developed with 3,3’-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Nichirei) at room temperature for 5–7 min. The sections were counterstained with hematoxilin.

For immunofluorescent histochemistry, the brains were removed from three mice for all groups, post-fixed overnight in 4% buffered PFA at 4 °C after perfusion, and then cryoprotected in 30% sucrose. The brain was frozen in powdered dry ice, and 50-µm-thick floating sections were prepared using a Cryostat (CM3050 S; Leica Biosystems, Nussloch, Germany). The sections were blocked and permeabilized with PBS including 10% normal goat serum (Sigma Aldrich) and 0.3% Triton-X 100 for 1 h at room temperature. The sections were then incubated with primary antibody (Anti-Iba1, 1/500; Wako; Anti-CD68, 1/200, Serotec/Bio-Rad, Raleigh, NC, USA; Anti-CD31, 1/100, Abcam, Cambridge, UK) for 3 h at room temperature, followed by secondary antibody (Anti-rat IgG, Alexa488, 1/200; Anti-rabbit IgG, Alexa568, 1/200; Molecular Probes-Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature in the dark. The sections were then mounted on slide glass with DAPI-Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Confocal images were obtained using a Zeiss LSM700 confocal fluorescence microscope equipped with diode lasers (405, 488, and 568 nm; Carl Zeiss, Oberkochen, Germany). The images were processed using the accompanying Zen image acquisition software package (Carl Zeiss). The histological data were represented as typical micrographs of the first SRS-induced mice at day 7 after SE.

4.7. Measurement of IL-1β, IL-6 and angiopoietin-2 in hippocampus of post-SE mice

The frozen whole hippocampus was weighed and homogenized in 5× volume of extraction buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM PMSE, 0.05% Tween20, and 1% protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan). The samples were centrifuged at 1000g for 10 min at 4 °C, and then the supernatant was collected and centrifuged at 20,000g for 40 min at 4 °C to remove any remaining debris. The protein concentration was measured with a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). The concentrations of mouse angiopoietin-2 and IL-6 in 25 µL of each brain supernatant were measured using a Milliplex™ MAP Mouse CVD Magnetic Bead Panel 2 (Millipore, Billerica, MA, USA) in accordance with the manufacturer’s instructions. Mouse IL-1β levels were measured using a commercially available ELISA kit (mouse IL-1β ELISA Ready-SET-Go! ; eBioscience-Affymetrix, San Diego, CA, USA) in accordance with the manufacturer’s instructions. The concentrations of each analyte in the brain samples were normalized to the total protein concentration (n=3 for all groups). The expressions of cytokines were included with the data of the first SRS-induced mice between days 7 and 10 after SE.

4.8. Statistical analysis

All data are shown as the mean ± standard deviation (SD) or as the median and box plots showing the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as the upper and lower error bars plus individual outliers. The BellCurve for Excel software program (Social Survey Res. Info. Co., Ltd. Tokyo, Japan) was used to perform the Kruskal Wallis post-hoc Steel or Steel-Dwass test. A p-value less than 0.05 was considered to be statistically significant.

Conflicts of interest

The authors declare that there are no potential conflicts of interest related to the present manuscript.

Acknowledgements

This work was supported by JSPS KAKENHI Grant number JP16K10216 (to K.I.), JP15K18947 (to R.K.) JP15K08122 (to H.N.) and JP26740024 (to Y. I.) and was financially supported in part by Tokushima Bunri University. This manuscript has been checked by a

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Reverse primer sequence</th>
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<td>TNF-α</td>
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<td>IL-6</td>
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<td>HIF-1α</td>
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<td>AGGGAATAAATCAATGGC</td>
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<td>β-actin</td>
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<td>GGCGTACTTCGAGGCTCAGGA</td>
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</table>

All mRNA primers are listed in the 5′→3′ direction.

The expression data of cytokines were included with the data of the first SRS-induced mice between days 7 and 10 after SE.
Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainres.2016.09.038.

References


